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Repetitive Transcranial Magnetic Stimulation Reduces Neuronal Loss and Neuroinflammation in Parkinson's Disease Through the miR-195a-5p/CREB Axis

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ABSTRACT

AIM: To elucidate the molecular mechanism underlying the repetitive transcranial magnetic stimulation (rTMS) -induced improvement in Parkinson's disease (PD).

MATERIAL and METHODS: We established a PD model by administering 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine to SAMP8 mice. The mice were then subjected to rTMS. Motor coordination and cognition were assessed using rotarod and Morris water maze tests, respectively. Nissl staining was performed to evaluate neuronal apoptosis. Furthermore, western blotting was employed to assess the expression of tyrosine hydroxylase and brain-derived neurotrophic factor. Additionally, the levels of tumor necrosis factor-α, interferon-γ, and interleukin-6 in the cerebrospinal fluid were evaluated using specific enzyme-linked immunosorbent assay kits. The expression of miR-195a-5p and cyclic AMP-response element-binding protein (CREB) was examined using quantitative real-time polymerase chain reaction and western blotting. Dual-luciferase reporter assay was performed using primary cortical rat neurons to validate the interaction between miR-195a-5p and CREB.

RESULTS: rTMS improved cognition and motor coordination as well as reduced neuronal apoptosis/ and the levels of inflammatory factors in PD mice. It downregulated the expression of miR-195a-5p but upregulated that of CREB. In primary rat cortical neurons, miR-195a-5p directly targeted CREB, and we found that miR-195a-5p suppression enhanced cognitive and motor functions in PD mice. Moreover, miR-195a-5p downregulation decreased inflammatory response and neuronal loss in the PD mice.

CONCLUSION: rTMS exerted its neuroprotective effects on PD mice by regulating the miR-195a-5p/CREB axis. This finding reveals a novel mechanism through which rTMS improves PD and indicates that miR-195a-5p is a potential therapeutic target for PD treatment.

KEYWORDS: Repetitive transcranial magnetic stimulation, Parkinson's disease, miR-195a-5p, CREB

ABBREVIATIONS: PD: Parkinson's disease, **rTMS:** Repetitive transcranial magnetic stimulation, **CREB:** Cyclic AMP-response element-binding protein, **SN:** Substantia nigra, **NC:** negative control, **SD:** Standard deviation, **ANOVA:** Analysis of variance, **TNF:** Tumor necrosis factor, **IFN:** Interferon, **IL:** Interleukin

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INTRODUCTION

The incidence of Parkinson's disease (PD) is estimated to be 5–35 cases/100,000 individuals/year (28), with a mortality rate that increases with age. PD is mainly characterized by bradykinesia, resting tremor, rigidity, and cognitive impairment, which severely limit the quality of life of the patients (24). Pathologically, significant neuronal apoptosis and α-synuclein accumulation can be observed in the substantia nigra (SN) (7). However, the underlying pathophysiological mechanism remains unknown. It has been postulated that the expression of α-synuclein can result in mitochondrial dysfunction, impaired autophagy, and neuronal apoptosis (9,24). Furthermore, a high level of oxidative stress and neuroinflammation are known to contribute to PD (17,32). Current therapeutic options include the administration of levodopa formulations, dopamine receptor agonists, monoamine oxidase-B inhibitors, and catechol-O-methyltransferase inhibitors, and the treatment of non-motor symptoms (1). However, these treatments are known to cause side effects.

Notably, a few novel strategies, such as the transplantation of stem cells into the striatum, gene therapy, and deep brain stimulation, have been developed to improve the clinical outcomes of patients with PD (17). Chou et al. reported that repetitive transcranial magnetic stimulation (rTMS) could enhance motor function in patients with PD (6). Additionally, rTMS can improve cognitive function and depressive symptoms (13,31). Lee et al. demonstrated that rTMS improves the survival of dopamine neurons (18). Nevertheless, the molecular mechanism through which rTMS improves PD remains elusive. It has been reported that rTMS promotes the proliferation of neural stem cells by regulating the expression of miR-25 (12). In addition, several studies have revealed that microRNAs regulate the pathogenesis of PD, and changes in the microRNA expression profile have been observed in patients with PD (11). Ding et al. identified five microRNAs that could serve as biomarkers for PD (8). One of these microRNAs is miR-185, which can serve as a therapeutic target for PD because of its inhibitory effect on neuronal apoptosis (33). Accordingly, we assumed that rTMS can influence the microRNA expression profile to exert its neuroprotective effects on PD.

Herein, we hypothesized that rTMS can protect neurons by regulating microRNA expression, and therefore, we established a PD rat model. Our findings included the following: 1) rTMS can ameliorate cognitive impairment and motor dysfunction in PD mice, 2) rTMS can downregulate miR-195a-5p expression and upregulate cyclic AMP-response element-binding protein (CREB) expression; and 3) miR-195a-5p can directly bind to CREB. These findings indicate that rTMS has the potential to improve PD by modulating the miR-195a-5p/CREB axis.

MATERIAL and METHODS

PD mice model and rTMS treatment

Animal experiments were approved by the Ethical Committee of our hospital, and experimental protocols were in accordance with the guidelines of the hospital for the care and use of experimental animals. Forty-eight male SAMP8 mice (20–30 g; 3 month-old) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. and maintained at our animal laboratory (temperature of 24 ± 1°C, relative humidity of 60 ± 10 %, and a 12-h light-dark cycle), with free access to food and water. After 1 week of adaptation, the mice (n=80) were randomly assigned to five groups (n=16 per group): 1) vehicle-treated control group (sham), 2) 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated PD group (PD), 3) MPTP and low rTMS co-treatment group (PD + L rTMS), 4) MPTP and high rTMS co-treatment group (PD + H rTMS), and 5) MPTP and miR-195a-5p antagomir co-treatment group (PD + miR-195a-5p antagomir). For PD induction, the mice were intraperitoneally administered MPTP (30 mg/kg) dissolved in phosphate-buffered saline for 7 days, consecutively. After successfully inducing PD, rTMS was performed as previously described (2). The mice were subjected to low-frequency (1 Hz) or high-frequency (10 Hz) rTMS, and the intensity of stimulation was set to 1.3 T. Two sessions of rTMS, comprising 1000 pulses in 10 trains, were performed daily for 3 weeks.

Rotarod test

The rotarod test was conducted to evaluate the motor function of the experimental mice, as previously reported (19). Prior to the rotarod test, the mice were trained on a rotarod for 3 days, and the rotarod was accelerated based on the previously established protocol. After adaptation to the rotarod, the test protocol was performed, and the time latency to fall from the rotarod was recorded. Each test was performed in triplicate.

Morris water maze test

In this test, the mice were placed in a circular tank (80 cm in diameter, 40 cm in height) filled with blue-black ink and water to a depth of 29 cm. A circular platform (9.5 cm diameter, 28 cm height) was placed inside the circular tank. The mice escaped the maze by climbing onto the circular platform. Distal visual cues, which would allow the mice to identify the circular tank, were placed around the room. Cameras were installed near the tank to record the activities of the mice.

Nissl staining

The mouse brain tissue was appropriately collected, and the SN was isolated and dehydrated with dimethylbenzene and different ethanol concentrations. Next, the specimens were incubated with 0.5 % cresol purple at 24 °C for 10 min, followed by incubation with 0.25 % glacial acetic acid-ethanol solution. Finally, the prepared specimens were differentiated using various ethanol concentrations and mounted on a glass slide. The tissues were observed using a phase-contrast microscope (Olympus, Japan).

Cell culture and proliferation assay

Primary cortical neurons were isolated from Sprague-Dawley rats as previously described (17). They were cultured in Roswell Park Memorial Institute-1640 medium (Beyotime, Shanghai, China) containing 2 % B27 (Invitrogen, USA) and 10 % fetal bovine serum (Gibco, USA) and incubated at 37 °C under 5 % CO_2.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Trizol (Sigma-Aldrich, USA) was used for the extraction of total RNA, and miRNA was obtained using Molpure Cell/Tissue miRNA Kit (Yeasen, Shanghai, China). mRNA was transcribed using a Two-step RT-PCR Kit (TaKaRa, Japan), and TaqMan MicroRNA Reverse Transcription Kit (Invitrogen, USA) and SYBR green (Roche, Switzerland) were used to transcribe miRNA. U6 was used as the endogenous control for miR-195a-5p. For other primers, GAPDH was employed as the endogenous control. The primer sequences used in this experiment are shown in Table I.

Western blotting

Protein samples were obtained from lysates of primary rat cortical neurons and mouse brain tissue samples using RIPA lysis buffer (Beyotime, Shanghai, China). To measure protein expression levels, the obtained samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by transfer onto polyvinylidene difluoride membranes (Millipore, USA). After blocking with 5 % non-fat milk at 4 °C for 1 h, the membranes were incubated with primary antibodies against tyrosine hydroxylase (TH; 1:1000; Cell Signaling Pathway, Cat No: 58844), brain-derived neurotrophic factor (BDNF; 1:1000, ab179800, Abcam, UK), CREB (1:1000; Thermo Fischer Scientific, USA, Cat No: MA5-32090), and GAPDH (1:1000; ab8245, Abcam, UK) at 4 °C overnight, followed by incubation with the appropriate secondary antibody at room temperature for 1 h. GAPDH was used as the internal control. Protein bands were visualized using an enhanced chemiluminescence kit (Beyotime, Shanghai, China).

Table I: Primer Sequences

Gene	Primer 5'→3'
MiR-195a-5p	F: CACCCAACTCTCCTGGCTCTA
	R: CACCCAACTCTCCTGGCTCTA
U6	F: CCCTAGAGTAGGGAGACAGGG
	R: GAGGTGCCACGACATACGAC
CREB	F: CAGGGGTGCCAAGGATTGAAG
	R: ACTGCTAGTTTGGTAAATGGGG
GAPDH	F: AGGTCGGTGTGAACGGATTTG
	R: GGGGTCGTTGATGGCAACA

Dual-luciferase reporter assay

Briefly, primary rat cortical neurons were seeded onto 96-well plates. At 60 % confluence, the cells were transfected or co-transfected with CREB-3′UTR-WT, CREB-3′UTR-MUT, miR-195a-5p mimic, miR-195a-5p mimic negative control (NC), miR-195a-5p inhibitor, and miR-195a-5p inhibitor NC. After 48 h, luciferase activity was measured in accordance with the manufacturer's instructions (Promega, USA).

Statistical Analysis

All data analyses were performed using SPSS 20.0 (IBM Corp., Armonk, NY, USA). Data are presented as the means ± standard deviation (SD) of three independent experiments. Differences among multiple groups were examined using analysis of variance (ANOVA), followed by Dunnett's post-hoc test or two-way ANOVA with Bonferroni's post-tests. Two-tailed $p<0.05$ was established as the threshold for statistical significance.

RESULTS

rTMS improves motor and cognitive functions in PD rodents

To simulate PD *in vivo*, mice were administered MPTP, and the rotarod test was performed to assess their motor functions. As illustrated in Figure 1A, MPTP treatment significantly decreased the time spent by the PD mice on the rotarod (retention time), compared with that spent by the mice in the sham group. However, rTMS increased the retention time of the PD mice, compared with that of the untreated PD mice. Moreover, treatment with 10 Hz rTMS demonstrated better efficacy than treatment with 1 Hz rTMS. As shown in Figure 1B and Table II, MPTP administration impaired the memory of the mice, and rTMS reduced the escape latency and reaction time of the PD mice. These findings indicate that rTMS can improve the outcomes of PD.

rTMS alleviates MPTP-induced neuronal damage and inflammatory response in PD mice

SN samples of the PD mice were subjected to Nissl staining

Table II: rTMS Improves the Motor and Cognitive Function of PD Rodents

	Learning ability		Memory ability	
Group	Reaction time (s)	Error time (s)	Reaction time (s)	Error time (s)
Sham	14.48 ± 1.82	3.46 ± 1.93	208.34 ± 24.67	3.96 ± 1.78
PD	68.92 ± 3.78***	7.89 ± 2.01*	58.56 ± 3.67***	7.89 ± 1.43*
PD + L rTMS	25.38 ± 3.45*	4.34 ± 1.29	175 ± 16.38	3.89 ± 1.23
PD + H rTMS	33.21 ± 2.13**	4.89 ± 0.78	152.23 ± 2.34*	5.93 ± 0.34

**p<0.05, **p<0.01, ***p<0.001 versus the sham control group.*

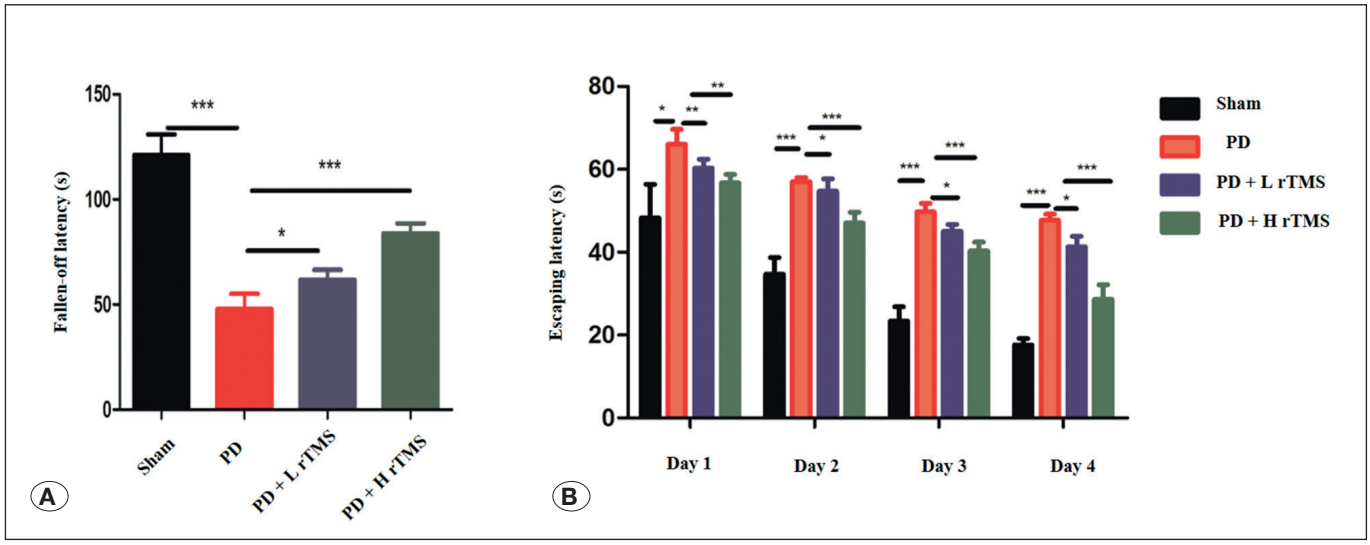

Figure 1: rTMS improves motor and cognitive functions in PD rodents. **A)** Retention time (rotarod test). **B)** Escape latency (Morris water test). NS, non-significant; *p<0.05, **p<0.01, ***p<0.001 by two-sided Student's *t*-test, *versus* the control group. PD, Parkinson's disease; rTMS, repetitive transcranial magnetic stimulation; L rTMS, low-frequency rTMS; H rTMS, high-frequency rTMS.

to evaluate the efficacy of rTMS. As shown in Figure 2A, MPTP treatment decreased the number of viable neurons in the SN of the mice, whereas rTMS increased neuronal viability. In addition, the expression of TH and BDNF was significantly decreased by MPTP, whereas rTMS upregulated the expression of these proteins (Figure 2B). Further, we evaluated the cytokine levels of the cerebrospinal fluid samples of the mice. We observed that MPTP administration significantly increased the production of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukin (IL)-6, whereas rTMS decreased the concentration of these inflammatory factors, indicating that rTMS could alleviate PD (Figure 2C-E). These findings indicated that rTMS could attenuate the MPTP-induced damage in the experimental rodents.

rTMS downregulates miR-195a-5p expression and upregulates CREB expression

miR-195 expression is associated with PD development (4, 8), and accordingly, we evaluated the expression of this microRNA in the present study. The expression of miR-195a-5p was downregulated in PD mice treated with rTMS (Figure 3A). Using StarBase (http://starbase.sysu.edu.cn/index.php), we discovered that miR-195a-5p, one of the fragments of miR-195, could bind to CREB (Figure 3B). In addition, we found that CREB expression was upregulated in PD mice treated with rTMS (Figure 3C, D). A dual-luciferase reporter assay was performed to confirm the interaction between miR-195a-5p and CREB. Primary rat neurons were transfected with the pGL3-CREB-WT luciferase construct as well as miR-195a-5p mimic, mimic NC, inhibitor, or inhibitor NC. Co-transfection with the pGL3-CREB-WT luciferase construct and miR-195a-5p mimic decreased luciferase activity, whereas co-transfection with the pGL3-CREB-WT luciferase construct and miR-195a-5p inhibitor increased luciferase activity (Figure 3E). Furthermore, the cells were transfected with pGL3-CREB-MUT luciferase construct and miR-195a-5p mimic, mimic NC, inhibitor, or inhibitor NC. Co-transfection with the pGL3-CREB-MUT luciferase construct and miR-195a-5p mimic or miR-195a-5p inhibitor did not influence luciferase activity (Figure 3F). Moreover, we observed that miR-195a-5p mimic downregulated CREB expression and miR-195a-5p inhibitor upregulated the expression of this protein when primary rat neurons were transfected with miR-195a-5p mimic NC, mimic, inhibitor NC, and inhibitor (Figure 3G). Therefore, we assumed that, in rodents, rTMS might exert its protective effects through the miR-195a-5p/CREB axis.

miR-195a-5p inhibition improves motor and cognitive functions in PD mice

Next, to confirm our previous findings *in vivo*, PD mice were treated with miR-195a-5p antagomir. We observed that treatment with miR-195a-5p antagomir alone prolonged the retention time of the PD mice in the rotarod test (Figure 4A). Moreover, the suppression of miR-195a-5p expression decreased the escape latency of the PD mice (Figure 4B and Table III). Thus, miR-195a-5p inhibition can exert protective effects on PD mice.

Suppression of miR-195a-5p expression in PD mice attenuates MPTP-induced neuronal damage and inflammation

Nissl staining and western blotting were performed to examine the impact of miR-195a-5p on PD. The results of qRT-PCR showed that the miR-195a-5p antagomir downregulated miR-195a-5p expression, compared with that observed in the PD group (Figure 5A). Moreover, the miR-195a-5p antagomir treatment increased the number of viable neurons in PD mice, compared with that observed in the untreated PD mice (Figure 5B). In addition, the suppression of miR-195a-5p expression in PD mice increased the expression of TH, BDNF, and CREB

Table III: Suppressing miR-195a-5p Expression in PD Rats Attenuates MPTP-Induced Neuronal Damage and Inflammation

	Learning ability		Memory ability	
Group	Reaction time (s)	Error time (s)	Reaction time (s)	Error time (s)
PD	70.82 ± 2.98	8.92 ± 3.02	60.21 ± 5.68	4.78 ± 0.68
PD + miR-195a-5p antogomir	34.21 ± 2.13***	5.02 ± 0.97	148.23 ± 3.46***	6.03 ± 0.67*

**p<0.05, **p<0.01, ***p<0.001 versus the sham control group*

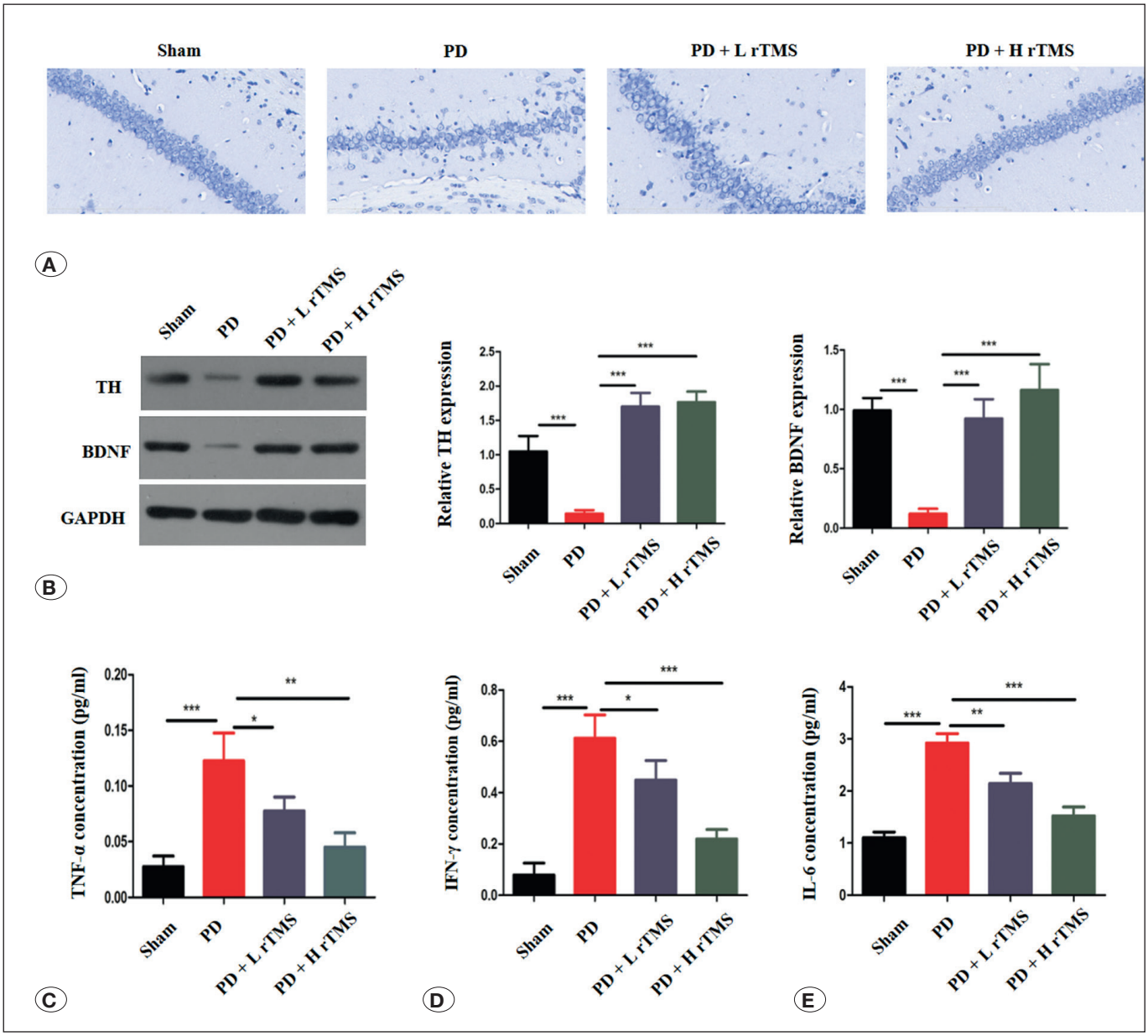

Figure 2: rTMS alleviates MPTP-induced neuronal damage and inflammatory response in PD mice. **A)** Nissl staining of SN section. **B)** Western blots showing TH and BDNF expression. **C)** TNF-α concentration. **D)** IFN-γ concentration. **E)** IL-6 concentration. NS, non-significant; *p<0.05, **p<0.01, ***p<0.001 by two-sided Student's *t*-test, *versus* the control group. rTMS, repetitive transcranial magnetic stimulation; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; L rTMS, low-frequency rTMS; H rTMS, high-frequency rTMS; TH, tyrosine hydroxylase; BDNF, brain-derived neurotrophic factor; TNF-α, tumor necrosis factor-alpha; IFN-γ, interferon-gamma; IL-6, interleukin-6.

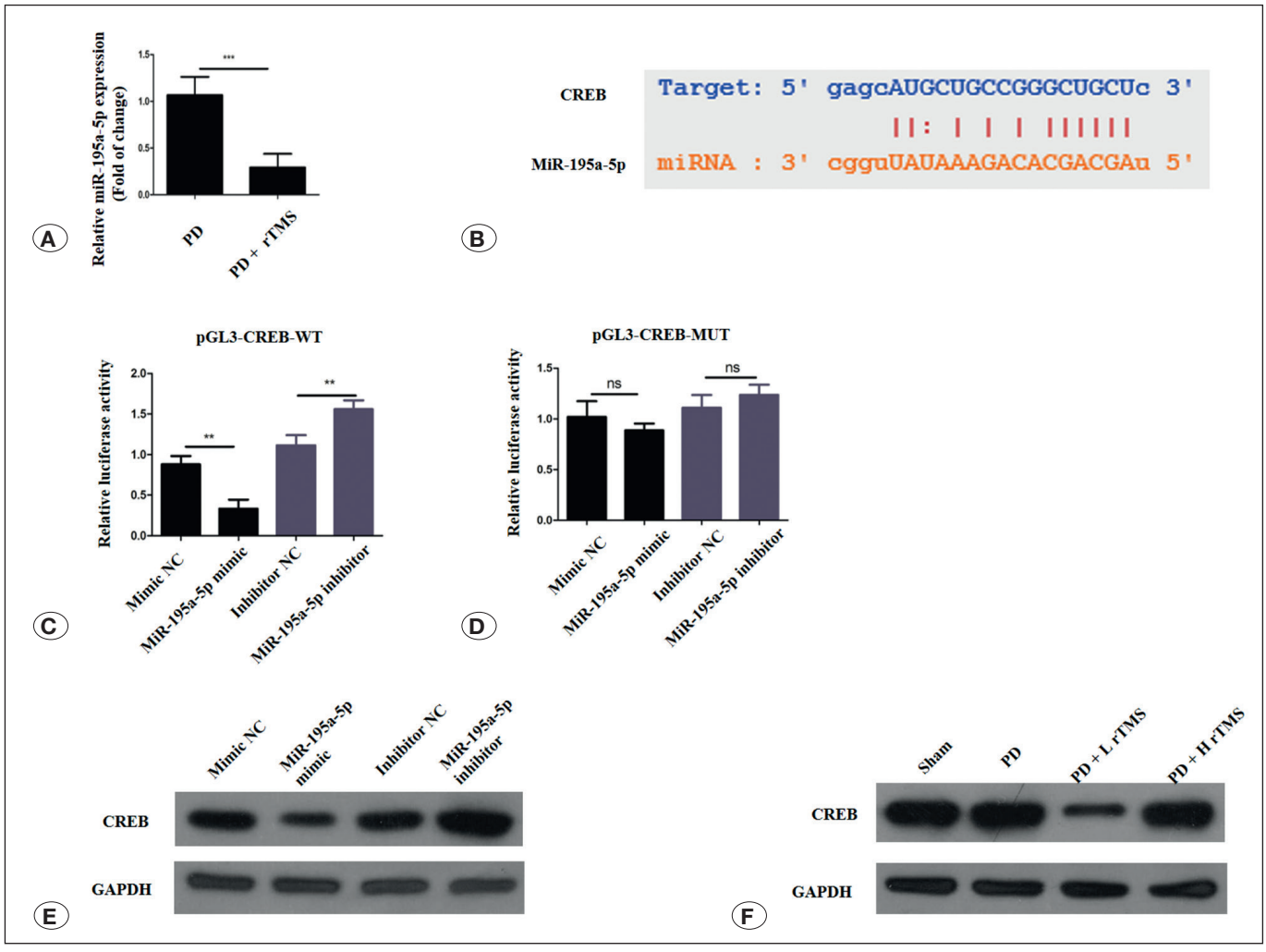

Figure 3: rTMS downregulates miR-195a-5p expression and upregulates CREB expression. **A)** miR-195a-5p expression. **B)** Illustration of the complementary sequence between miR-195a-5p and CREB. **C)** CREB activity. **D)** Relative luciferase activity. **E)** Relative luciferase activity. **F)** CREB protein expression. NS, non-significant; *p<0.05, **p<0.01, ***p<0.001 by two-sided Student's *t*-test, *versus* the control group. rTMS, repetitive transcranial magnetic stimulation; PD, Parkinson's disease; CREB, cyclic AMP-response element-binding protein.

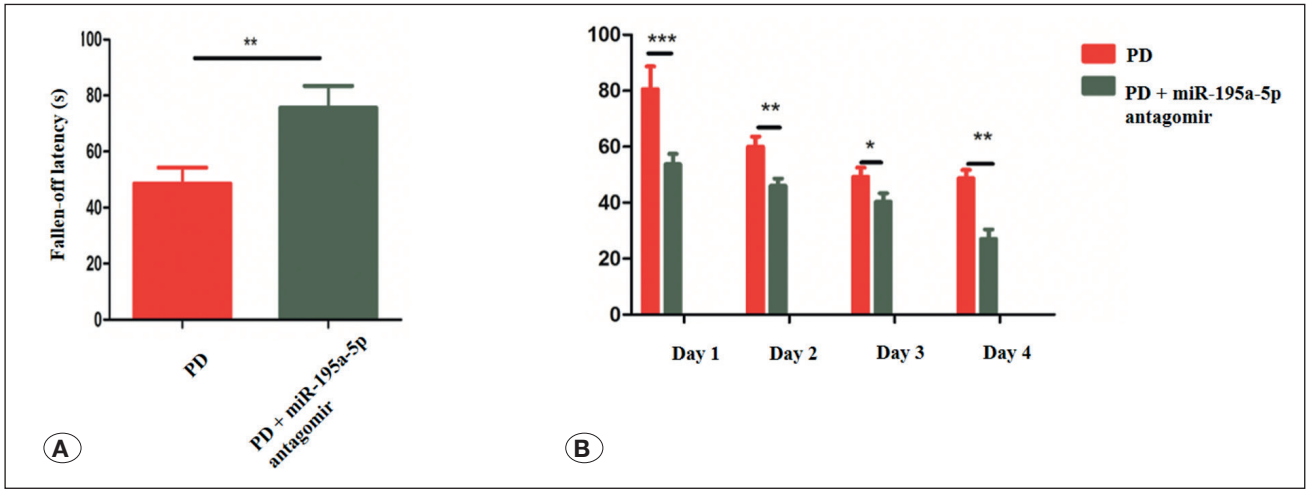

Figure 4: Inhibition of miR-195a-5p expression improves motor and cognitive functions in PD rats. **A)** Retention time (rotarod test). **B)** Escape latency (Morris water test). NS, non-significant; *p<0.05, **p<0.01, ***p<0.001 by two-sided Student's *t*-test, *versus* the control group. PD, Parkinson's disease.

(Figure 5C). Moreover, the downregulation of miR-195a-5p expression markedly reduced the expression of TNF-α, IFN-γ, and IL-6 (Figure 5D-F). Therefore, we demonstrated that at the molecular level, the downregulation of miR-195a-5p expression reduces neuronal damage in PD mice.

DISCUSSION

In the present study, we aimed to elucidate the mechanism through which rTMS improves motor and cognitive functions in patients with PD. Accordingly, a suitable PD mouse model was established, followed by rTMS treatment. By employing the rotarod and Morris water maze tests, we demonstrated that rTMS, especially high-frequency rTMS, could significantly improve motor and cognitive functions in PD mice, which was consistent with previous reports (30,34). Moreover, we observed that rTMS reduced neuronal apoptosis and inflammatory response in the SN. The primary pathological characteristic of PD is neuronal loss in the SN, which results

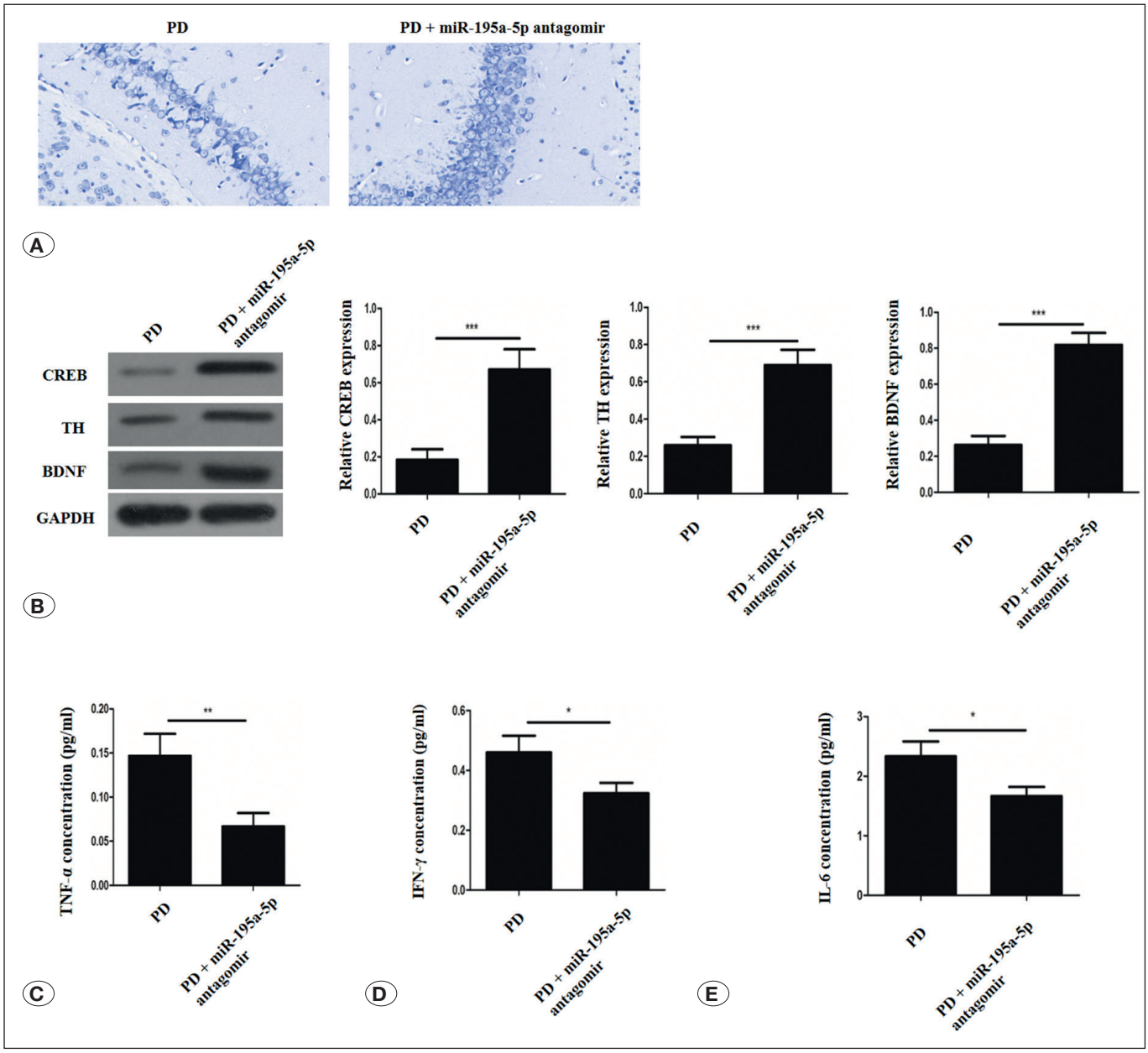

Figure 5: Suppression of miR-195a-5p expression in PD rats attenuates MPTP-induced neuronal damage and inflammation. **A)** miR-195a-5p expression. **B)** Nissl staining. **C)** Western blots showing the expression of CREB, TH, and BDNF. **D)** TNF-α concentration. **E)** IFN-γ concentration. **F)** IL-6 concentration. NS, non-significant; *p<0.05, **p<0.01, ***p<0.001 by two-sided Student's *t*-test, *versus* the control group. PD, Parkinson's disease; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; TH, tyrosine hydroxylase; BDNF, brain derived neurotrophic factor; CREB, cyclic AMP-response element-binding protein; TNF-α, tumor necrosis factor-alpha; IFN-γ, interferon-gamma; IL-6, interleukin-6.

in the dopaminergic denervation of the striatum (7). Therefore, restoring neuronal viability in the SN is considered an important therapeutic strategy for PD. Furthermore, the SN demonstrates persistent inflammation in PD, which is one of the factors involved in the development of this disease. For instance, the serum concentration of TNF-α is associated with the severity of PD, facilitating the recruitment of immune cells and promoting oxidative burst (16). In addition, TNF-α stimulates the apoptosis of dopaminergic neurons by activating the mitochondrial complex (25). Moreover, there appears to be a link between IFN-γ signaling and the accumulation of α-synuclein. IFN-γ can act with TNF-α to activate microglial and astroglial cells, which can result in persistent neuroinflammation (3,9,21). IL-6 concentration in the cerebrospinal fluid can be used as a biomarker of PD severity (10,15), and this cytokine can propagate the inflammatory response (27, 29). Thus, suppressing inflammatory responses in patients with PD is considered a new therapeutic direction for this disease. In the present study, we observed that rTMS could reduce neuronal loss and inflammation.

In addition, rTMS decreased the expression of miR-195a-5p and increased that of CREB. Although only a few studies have investigated miR-195a-5p and its role in the pathogenesis of PD, the roles of miR-195 have been explored by several studies. miR-195 expression is upregulated in patients with PD; hence, it can be used for the diagnosis of early-onset PD (4,8). Moreover, Ren et al. reported that miR-195 could trigger inflammation in PD by regulating the expression of Rho-associated kinase 1 (26). The present study reveals that in a PD mouse model, suppressing the expression of miR-195a-5p (a fragment of miR-195) can alleviate motor and cognition impairments as well as decrease neuronal loss and inflammation. Thus, the inhibition of miR-195a-5p expression demonstrates significant therapeutic potential.

Furthermore, we found that miR-195a-5p could bind to CREB, and this mediated the neuroprotective effects of rTMS. Notably, to the best of our knowledge, the present study is the first to report the association between miR-195a-5p and CREB. CREB can regulate apoptosis and inflammation. It has been reported that the activation of the CREB signaling pathway can reduce neuronal apoptosis in a PD rodent model (20). Some studies have demonstrated that CREB can upregulate BCL-2 and BDNF expression to prevent cell death (14,35). Notably, it can cooperate with BDNF to ameliorate neuroinflammation mediated by astrocytes (36). In addition, CREB demonstrates an immunosuppressive effect on immune cells by interacting with the TORC1 signaling pathway (22). Moreover, it is critical for neurogenesis, memory consolidation, cognition, and cortical circuit plasticity (5,22,23,37). In the present study, rTMS treatment upregulated CREB expression, and we speculate that CREB mediated the neuroprotective effects of rTMS.

Nonetheless, our study had a few limitations. First, our study presents preliminary findings and further clinical studies are needed to validate our results. Second, while our findings suggest that rTMS might influence the microRNA expression profile in PD mice, and miR-195a-5p may be one of the most important microRNAs regulating PD, the underlying molecular mechanisms are not yet clear, and require further in-depth bioinformatic analysis and experiments.

CONCLUSION

In conclusion, our findings showed that rTMS substantially alleviated motor and cognitive dysfunctions in PD mice, reducing neuroinflammation and neuronal loss. These effects were mediated by the modulation of the miR-195a-5p/CREB axis.

AUTHORSHIP CONTRIBUTION

Study conception and design: LS, FW, JD
Data collection: JH, LB
Analysis and interpretation of results: JH, LB
Draft manuscript preparation: LS, FW
Critical revision of the article: JD
All authors (LS, FW, JH, LB, JD) reviewed the results and approved the final version of the manuscript.

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